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13. ABSTRACT ***(Maximum 200 Words)***

We have proposed that one function of gap junctions is to transmit growth control signals. There is abundant evidence that most cancers, including breast cancers, lose the capacity to junctionally communicate and we hypothesize that this in part is responsible for their aberrant behavior. To test this hypothesis rigorously we have genetically engineered human breast cancer cells to contain the gap junction gene connexin 43 under the control of the tetracycline-inducible promoter. In the first year of this grant application, we genetically engineered MDA-435 cells to express connexin 43 under the control of a tetracycline-inducible promoter. We demonstrated that on removal of doxycycline, cells rapidly express connexin 43, integrated into the plasma membrane and became junctionally competent. We now demonstrate that upon induction of this gene, several aspects of growth control of changed: 1/. the colony forming ability in suspension culture is reduced by approximately 80% in contrast to non-induced cells; 2/. The saturation density of cells is reduced and this reduction is accompanied by an increased number of cells in the G2/M phase of the cell cycle. Molecular analysis of these growth-inhibited cells indicates an increased content of p21, an inhibitor of Chinese is required for cell cycle transit. These results demonstrate that important aspects of growth regulation are modified by restoration of junctional communication and put us in a good position to identify the signals being communicated and the molecular mechanism of action.

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Introduction:
We propose to chemically identify the junctionally transmitted signals which we hypothesize to be responsible for the observed growth inhibition of breast tumor cells when in junctional communication with growth inhibited normal cells.

1/. Research accomplishments.

Technical objective 1 a. Production of genetically engineered cells containing connexin 43 under the control of a tetracycline-inducible promoter. This has been achieved as documented in Figure 1. We now possess 2 well characterized clones in terms of connexin 43 expression, and have cryopreserved additional clones for future use if needed. The properties of these clones are now being evaluated.

Technical objective 1b. Development of in vitro protocols for the delivery of the growth inhibitory signal from quiescent cells to junctionally competent breast cancer cells.

As the first stage of this analysis we are evaluating the response of the genetically engineered MDA-435 cells to junctional communication with themselves. As a rationale for these studies, we have previously demonstrated that several tumor cells enhance their degree of growth control when communication is re-expressed by pharmacological or genetic means.

Anchorage independent growth: In the present situation utilizing breast carcinoma cells, we have shown that the ability to grow in semi-solid agarose, as anchorage-independent colonies, is strongly suppressed in cells expressing connexin 43. These genetically engineered cells on normally grown in doxycycline to suppress connexin 43 expression and prevent the clonal selection of cells resistant to junctionally mediated growth inhibition. Cells from a single such inhibited culture were divided and either maintained in doxycycline two repressed connexin 43 gene expression, or were plated in doxycycline free medium. After 48 hr, when previous studies have shown strong of regulation of connexin 43 expression, and both groups of cells were trypsinized and plated at identical cell densities into semi-solid medium. Cultures were maintained for an additional 4 weeks with weekly editions of small volumes of growth medium to the surface of the semi solid agarose. Cultures were then stained with a vital dye to distinguish living from dead cells, and the number of colonies counted manually. Non-induced cultures produced many large colonies under these conditions, the expected behavior of fully malignant cells, while induced colonies produced only a few such large colonies, while the majority of cells form colonies only approximately 8 cells in size. Thus, the expression of connexin 43 dramatically inhibits by about 90% the ability of cells to form large colonies. No effects were observed when we treated with doxycycline the parental MDA cells containing only the tetracycline receptor demonstrating that doxycycline itself is not responsible for this change in behavior. Thus, when cells are suspended, the induction of connexin 43 as profound effects on their ability to form colonies under these conditions; conditions which do not normally allow the growth of non-malignant cells.

Growth as monolayers: under conditions where we reduced the concentration of car serum in culture medium, we were able to show that connexin 43 induced MDA cells proven to marginally lower saturation densities than do non--induced cells (Figure 2). We have performed flow cytometry on these cultures utilizing the DNA stain, propidium iodide, and have shown in 4 experiments an increase in the portion of G2/M cells in

connexin induced cultures (Fig. 3). This was unexpected, since in most cells physiological cell cycle arrest occurs in the G1 phase of cell cycle. As with other assays, clone 16 responds most dramatically to induction. As can be seen in this figure, the percentage of G2/M cells increased from 9.9% to 26% on induction, with a proportional reduction in the number of G1 cells from 48.9 to 33.6. We are excited by these findings as they offer a tool to discover the molecular mechanisms of junctionally communicate growth arrest. We have begun preliminary molecular analyses of these induced cultures and using RT/PCR and have made the preliminary observation that the level of the cell cycle inhibitor p21 is increased in connexin 43 induced cultures. This makes be confirmed by Western blotting. We are extending our studies to look at other cell cycle regulating genes in order to obtain information on which genes change in the expression levels as a consequence of gap junctional communication. This information could the vital in aiding us identify the chemical nature of the junctionally transmitted signal (s).

Influence of connexin 43 expression on growth of MDA cells when in junctional communication with growth-inhibited normal cells. Because it may be expected that the MDA cells themselves produced only limited amounts of the putative messenger involved in junctionally transmitted growth control, we are currently searching for cell lines which are capable of communication with these breast carcinoma cells, are themselves growth inhibited and can transmit this growth inhibition to the MBA cells in which they are in junctional communication i.e. can act as donor cells. In other situations utilizing murine cells we have reported strong growth inhibition in these situations. We require this demonstration of response to junctional signals in order to move on to the next stage of this investigation; the identification of the signals themselves. We are currently experiencing difficulties in identifying a suitable donor cell line. The mouse fibroblasts cell lines we have previously utilized, while also expressing connexin 43, appear not capable of entering into junctional communication with human carcinoma cells as evidenced by lack of dye transfer from microinjected MDA cells to surrounding murine cells and no growth inhibition is seen in these co-cultures (Figure 4). This may be to the lack of suitable cell surface receptors. However, communication and growth inhibition is seen in co-culture with rat NRK cells (Figure 5). We prefer to have human/human co-cultures and have performed the same type of experiment utilizing immortalized human keratinocytes, the HaCat cell line, which grows to a growth arrested cell monolayer in defined serum-free conditions. Unfortunately, we have been unable to achieve growth of the MDA cells under the same conditions, while if we expose HaCaT cells to the medium utilized to grow MDA cells, the HaCaT cells terminally differentiate and are not suitable for use. We have recently obtained a line of immortalized human breast epithelial cells from Dr. Shay, and are currently evaluating their use under these experimental conditions. We propose to continue or search for compatible cell types, where one cell acts as donor of growth inhibitory molecules, while the other cell, in this case genetically engineered MBA cells, act as recipient. When this model system is in place i.e. we have cells which generate the signals and cells which respond in a predictable manner,, we will be in a much better situation to conduct experiments to identify these junctionally transmitted signals.

Technical objective 1c. Detection of the junctionally transfer signal in breast cancer cells by measurement of cell cycle related parameters. As detailed above, under growth in monolayers, we have begun the analysis of cell cycle changes induced by connexin expression. Once we have successfully identified donor cells capable of junctional communicating with these MDA cells, we will be in the position to: 1/. Determine the phase of the cell cycle inhibited by communication with normal cells and, 2/. Fractionate

these donor cells for the putative signal and, 3/. Have an assay system to measure the effects of the signals. Research in year three of the application will focus on these final objectives.

2/. Key research accomplishments;
Creation of tetracycline inducible MDA cell lines which reversibly express connexin 43.

Demonstration that these cells have dramatically reduced anchorage independent growth of on induction of connexin 43.

Demonstration that in monolayer cultures the G2/M phase of the cell cycle is extended in connexin 43 expressing cells.

Reportable outcomes:

Publications:
1/. Abstract submitted and poster presented at the Era of Hope meeting organized by the DOD in Atlanta Georgia.

2/. Abstract submitted to the American Society for Cell Biology to be held in San Francisco in December 2000. This abstract has been accepted for oral presentation at a gap junction subsection at this meeting.

Development of cell lines: we have developed an MDA 435 cell line containing the tetracycline promoter which will be a useful line for others who wish to create their own tetracycline inducible gene systems. We have also developed this line containing the promoter and a connexin 43 inducible gene.

Research training: This grant provides stipends for two Ph.D. students enrolled in the cell and molecular biology program that the University of Hawaii. It is expected that both students will conduct research of sufficient quality to qualify for their Ph.D.'s within the next two years.

Conclusions: our creation of a tetracycline-from connexin 43 inducible gene system in a human mammary carcinoma cell line offers significant advantages for the study of junctional communication over previously available models. Previous models utilized constitutively activated genes whose functions have to be inferred by comparison of differences between the transfected cells and parental cells. However, as we have recently discovered utilizing HeLa cells, there exist major clonal variations within recently cloned cell lines. This clonal variation encompasses changes in growth control and ability to form tumors. Thus, during the selection of constitutively active genes, clones may be selected which differ markedly in properties from the parental cells. This makes for great difficulties in interpreting the effects of the introduced gene. In contrast, use of inducible lines means that comparisons can be made between a single cell line in which the gene is or is not activated. This allows the unambiguous determination of the function this gene, since the inducing agent, doxycycline, is not known to have biological properties at the concentrations involved. Thus, our reports that there is an increase in G2 phase of the cell cycle can be confidently ascribed to the effects of connexin 43 expression rather than to clonal differences in the two cell lines compared. Moreover, in our projected research to discover the chemical identity of junctional transmitted signals, we will be in a position to add putative signals to cells either expressing, or not

expressing gap junctions, thus allowing any effects of the putative transmitter to only be seen in the context of effective junctional communication. This should effectively eliminate false positives, for example, compounds having non-specific effects on cell cycle transit time. With our possession of the cell line, and are demonstration of responses to connexin 43 induction, we now feel we are in an excellent position to attack the major goal of this application, the identification of junctional transmitted signals.

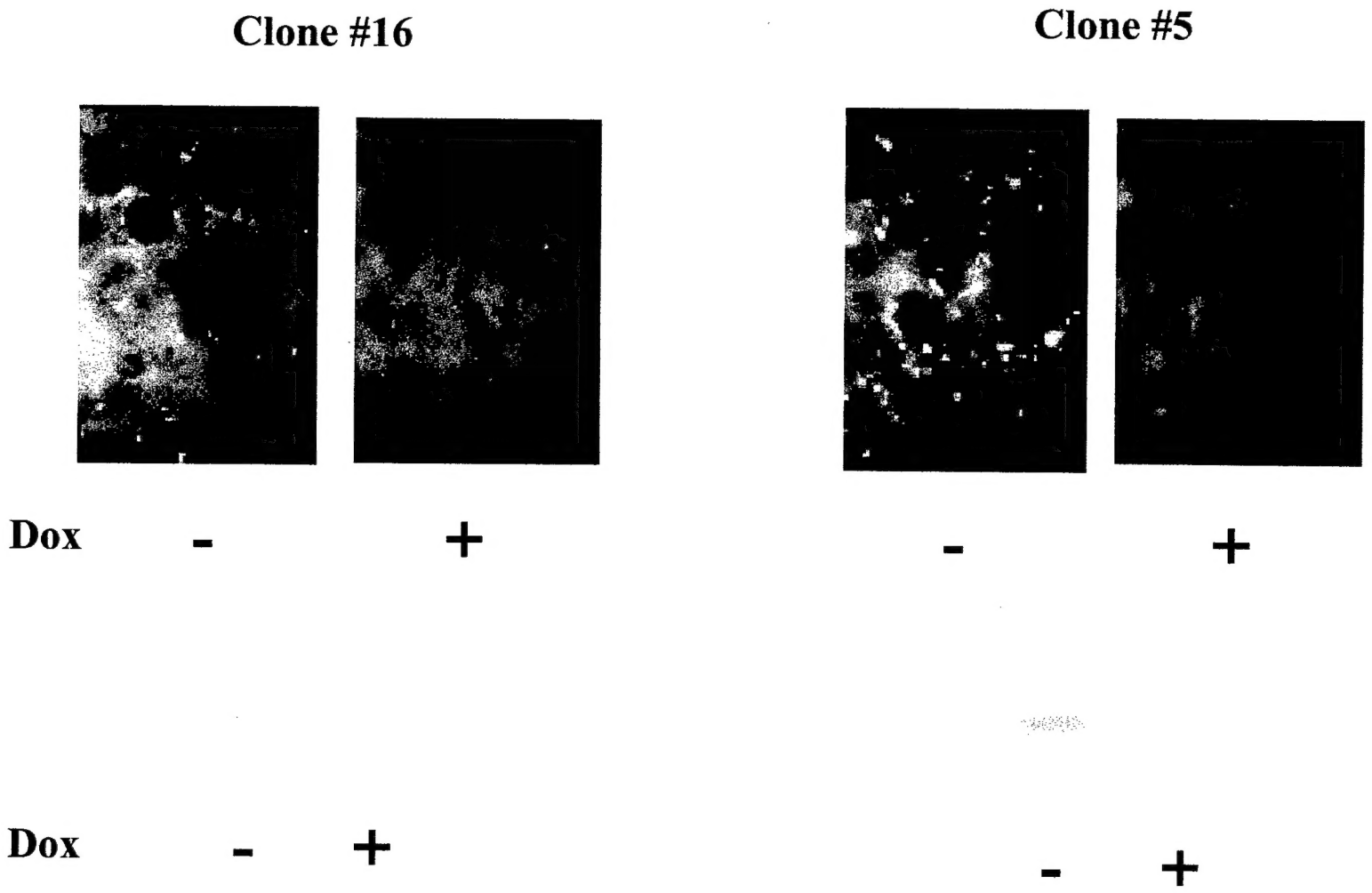
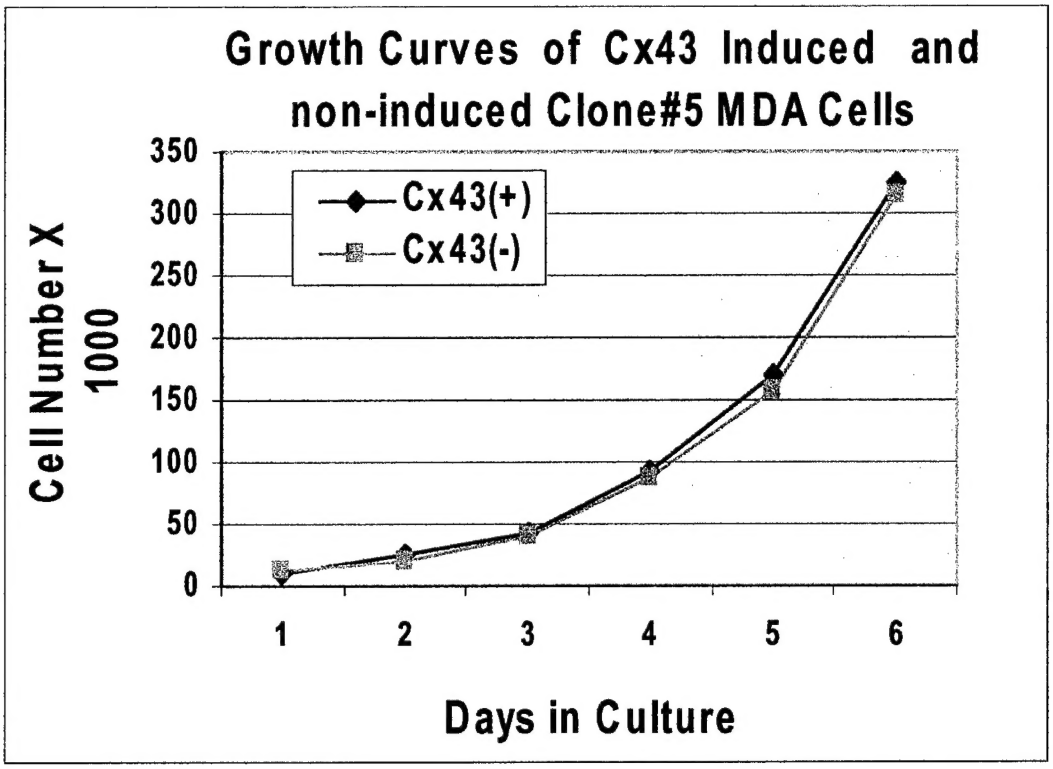


Fig1. Immunofluorescence staining (above) and western-blotting(below) analysis of Cx43 expression: clone #16 (left) and clone#5 (right) with (+)or without (-) doxycycline (Dox).

a



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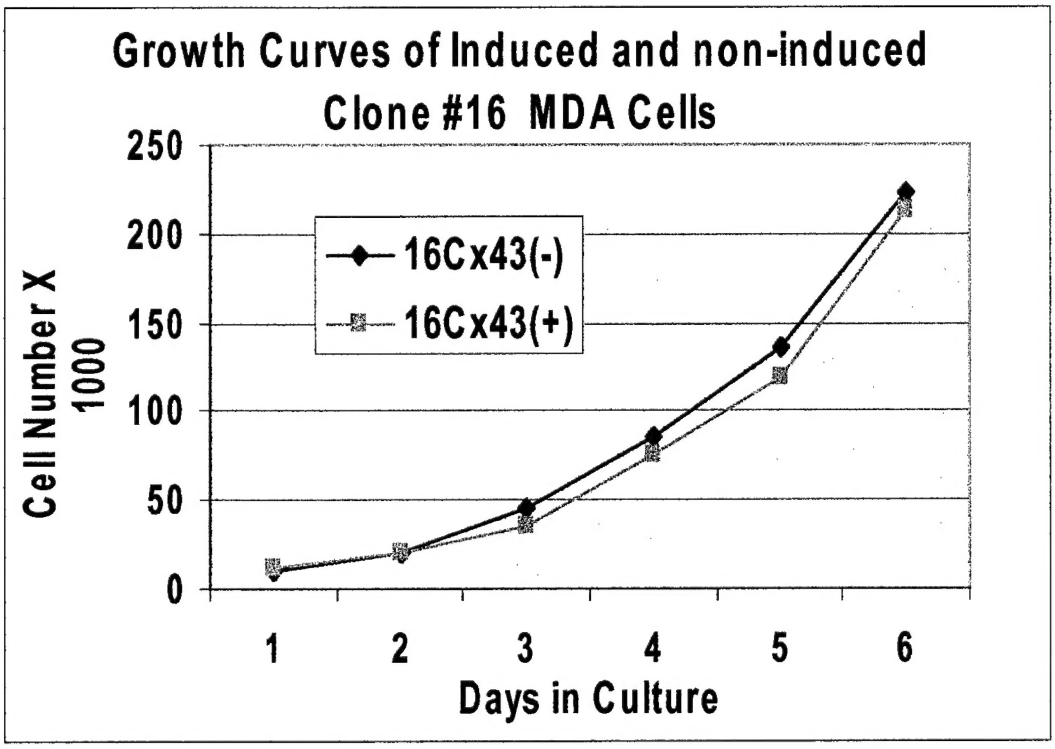
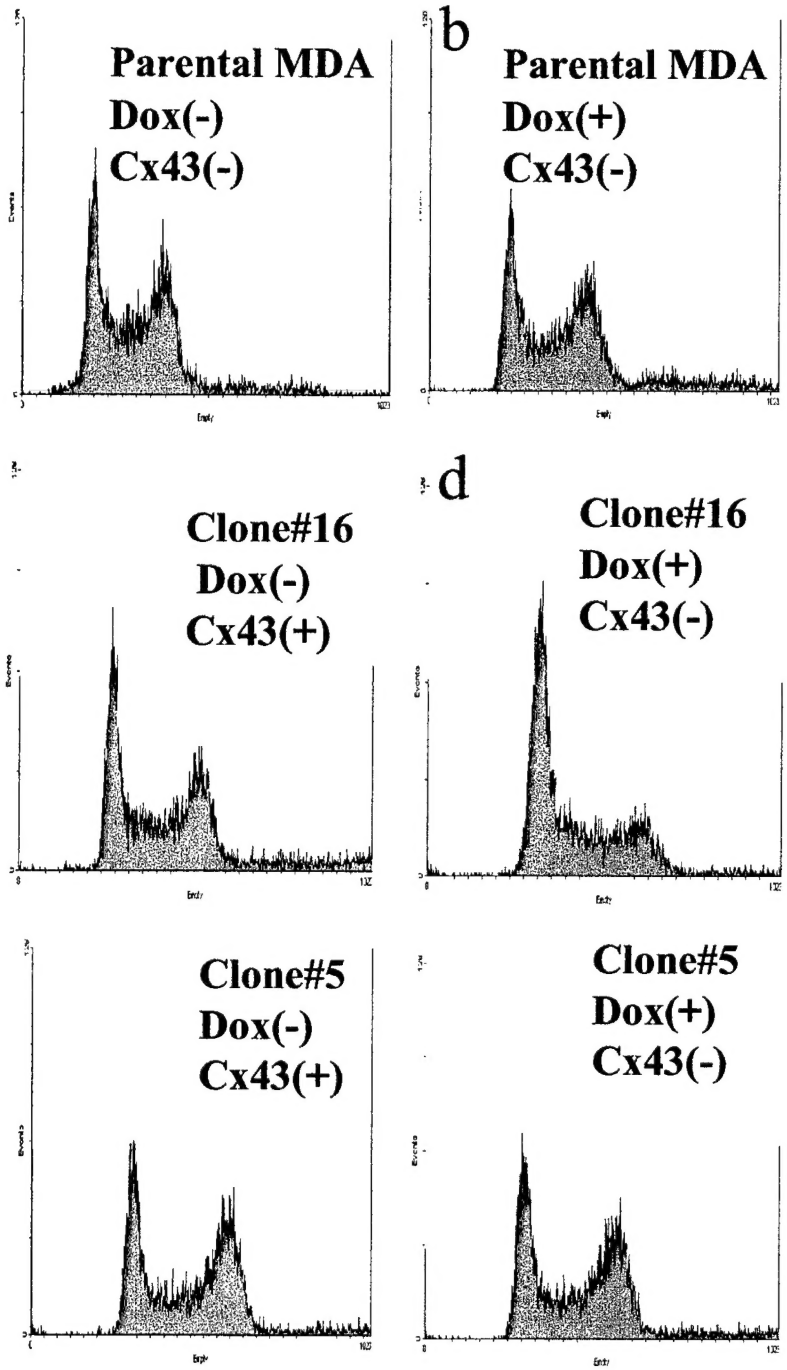


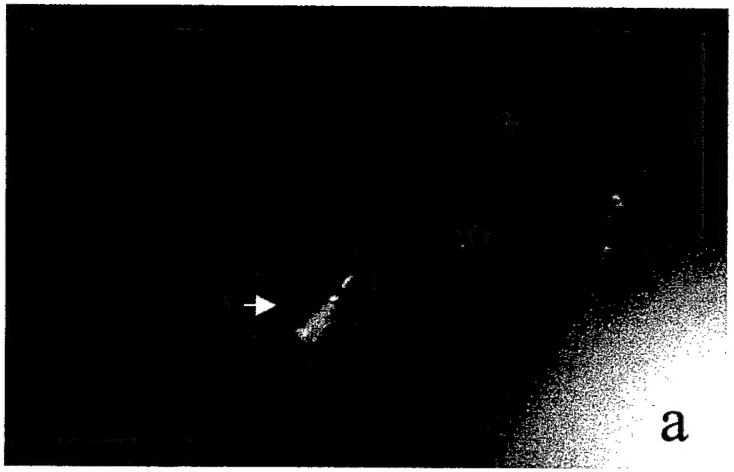
Fig 2. Growth curves of Cx43 induced and non-induced clone #5(a) and clone #16 (b) cells . Cells were seeded at 10^4 cells /well in a 24 well culture dish. Subsequently, duplicate wells were counted electronically every day.

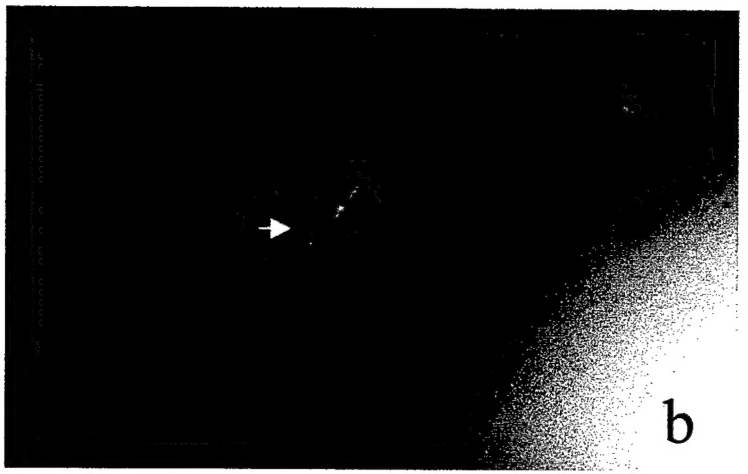


	G1/G0%	S%	G2+M%
a	25.06	49.97	24.95
b	26.73	46.49	26.78
c	33.62	40.37	26.01
d	48.91	41.15	9.94
e	28.17	38.56	33.27
f	28.40	41.99	29.62

Fig 3. Cell cycle analysis of parental MDA cells, panels a, b; Clone #16, panels c, d; and Clone#5, panels e, f. Cells were all seeded at 2X10^5 cells/ml, then cultured for 2 days with or without doxycline (Dox -, +) before FACS analysis.

Note: major increase in G2/M cells in Cx43 induced clone# 16 cells; clone#5 appears non-responsive.





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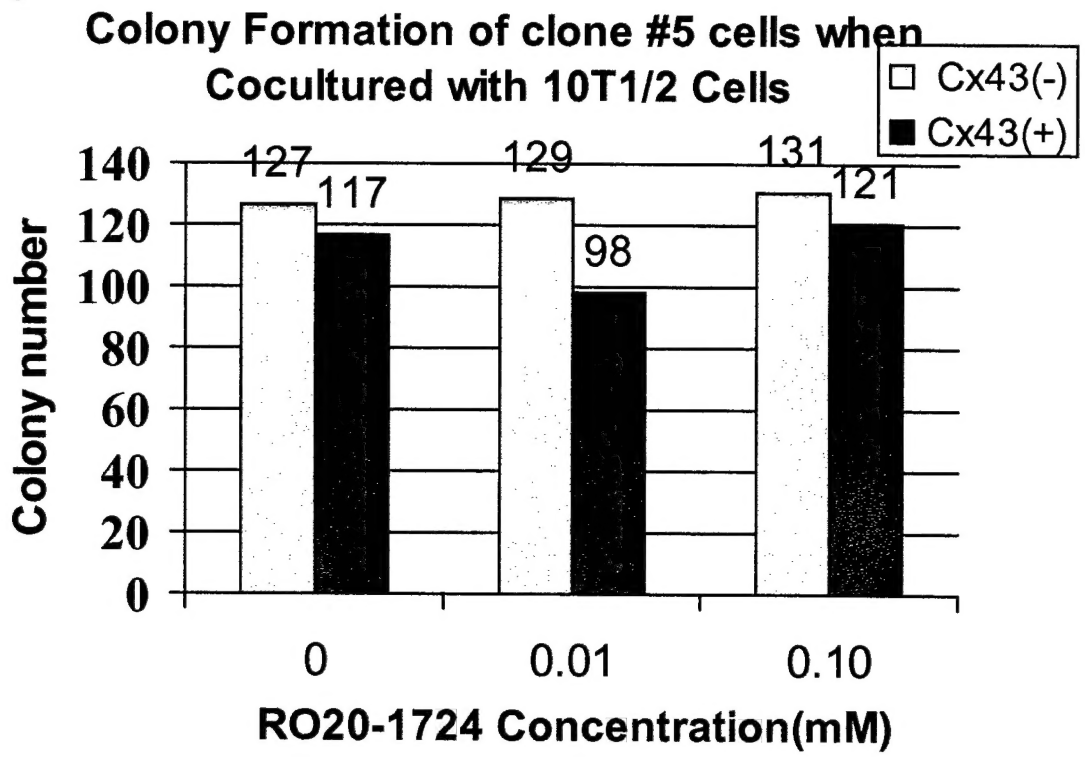
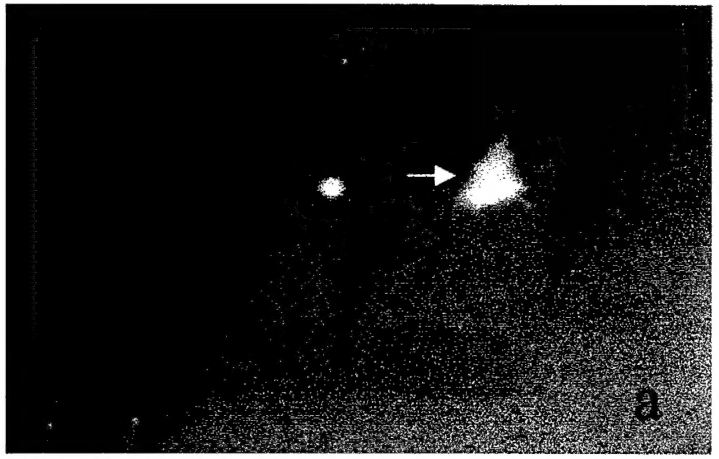
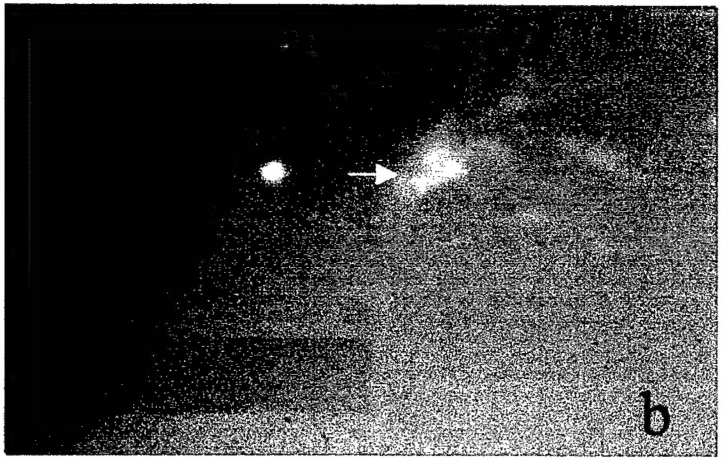


Fig 4. Clone# 5 were labeled with fluorescent microbeads and cocultured with confluent 10T1/2 cells. Results show microinjection of lucifer yellow into a labelled clone # 5 cell, after: panel a/ 0.5 min, and panel b/, 5 minutes. Note lack of transfer of dye to adjacent 10T1/2 cells. C. Lack of growth inhibition of Cx43 induced clone #5 cells by RO20-1724 when coculture with confluent 10T1/2 cells. 200 clone #5 cells /dish were plated onto 10T1/2 cells and treated after3 hours with the stated concentration of drug or acetone control.Cultures were fixed and stained after 7 days and scored for number of MDA foci. The suppression of colony formation was statistically insignificant $P>0.05$.





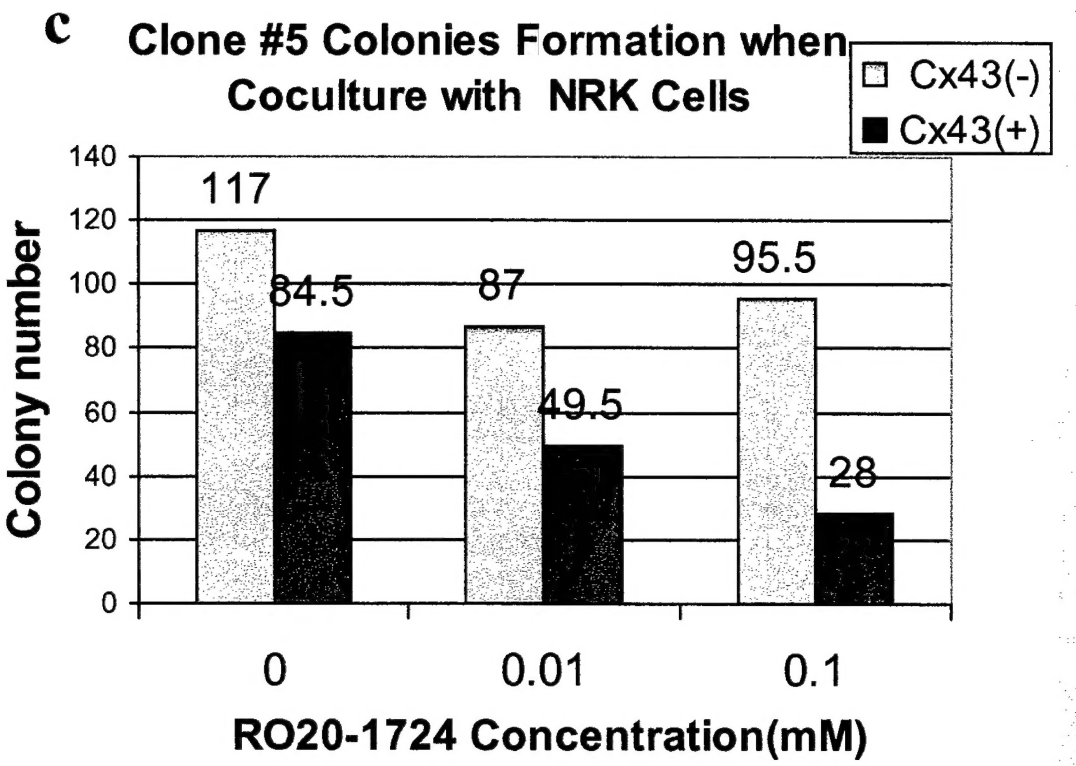


Fig 5. Coculture of clone# 5 cells, labelled with fluorescent microbeads, onto confluent NRK cells. A/ microinjection of lucifer yellow into a labelled clone # 5 cell 30 sec after injection; B/ 5 minutes later showing dye transfer from the labeled cell to adjoining unlabeled NRK cells. C. growth inhibition of clone #5 cells by RO20-1724 when in coculture with confluent NRK cells. 200 clone #5 cells /dish were plated onto NRK cells and treated after3 hours with the stated concentration of drug or acetone control .Cultures were fixed and stained after 7 days and scored for the number of transformed foci. Blue columns: non-induced; purple induced to express Cx43. The suppression of colony formation was statistically significant $P < 0.05$.